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14. ABSTRACT
This study was designed to determine the role of the fatty acid synthesis regulatory protein, Spot14 (S14) in mammary tumorigenesis. We have generated mouse models to identify effects of S14 overexpression and S14 loss on mammary tumor formation, growth, metabolism, and metastasis. To date, we have determined that S14 overexpression shortens mammary tumor latency, increases tumor fatty acid synthesis, but inhibits tumor metastasis. We found that tumors overexpressing S14 were well differentiated compared to control tumors, which likely explains their decreased metastatic ability. Conversely, loss of S14 did not affect tumor latency, but resulted in slower tumor growth rates. Furthermore, many of the tumors that formed lacking S14 had a cystic morphology. These tumors also had reduced fatty acid synthesis. Overall, these studies show that S14 plays an important role in mammary tumor growth and metabolism.

15. SUBJECT TERMS
mammary tumor, breast cancer, Spot 14, lipid synthesis

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Introduction:

THRSP/Spot14/S14 is a small cytoplasmic protein that is highly expressed in tissues that synthesize fatty acids de novo. This includes the liver, adipose tissue, and the lactating mammary gland. In the liver, S14 inhibits de novo fatty acid synthesis, but it is required in adipocytes for de novo fatty acid synthesis. S14-null lactating mice produce milk with reduced fatty acid content and nurse offspring with runted development. It has become increasingly clear that tumors display alterations in de novo fatty acid synthesis. Specifically, the enzyme fatty acid synthase (FASN) is often elevated in solid tumor types compared to surrounding normal tissue. High rates of fatty acid synthesis are thought to provide a survival, growth and metastatic advantage to cancer cells. S14 was reported to correlate with a poor patient outcome for women with breast cancer. Based on the role of fatty acid synthesis in cancer, the role of S14 in normal mammary biology, and the effect S14 potentially had on breast cancer outcome, we hypothesized that S14 overexpression in tumors would stimulate fatty acid synthesis, which would promote tumor growth and metastasis. Until this study, nobody had demonstrated a causal link between S14 and mammary tumorigenesis. To establish this link, we have generated a mouse model in which the MMTV promoter drives the mammary specific expression of S14. These mice have been crossed with well-characterized MMTV-Neu mice, which develop mammary tumors with a long latency. In this report, we summarize the studies conducted to date and demonstrate that S14 overexpression in MMTV-Neu mammary glands does indeed promote tumor formation and growth. Interestingly, tumors emerging from S14-transgenic mice are not metastatic. We have extensively profiled these tissues and have developed a proposed model of action for S14, based on the cell type in which we predict it to be expressed. In summary, we suggest that S14 is normally expressed in a well-differentiated cell type in the mammary epithelium, where it stimulates fatty acid synthesis for milk production under the control of prolactin. When prolactin is absent and oncogenic signaling is activated, we predict that the S14-mediated increase in fatty acid synthesis promotes cell proliferation, leading to tumor formation. Because S14 positive cells are well differentiated, the tumors that emerge are not metastatic. This model is supported by our transgenic mouse studies and by analysis of publicly available human breast cancer datasets.

Body:

I have described the studies performed to date in detail below, but to avoid formatting problems, I have chosen to put all of the described figures at the end of the document.

From SOW: Specific Aim 1*: Determine the effect of Spot14 loss on the growth and metabolism of mammary tumors in vivo in MMTV-Polyomavirus Middle T Antigen (PyMT) mice.*

Task 1 (Year 2 Months 9-12) We will generate MMTV-PyMT Spot14+/+ (Control) and MMTV-PyMT Spot14-/- (Spot14 null) female mice. These mice will be used for a tumor study, and have an average tumor latency of less than 8 weeks. We expect to generate enough females for our tumor study by the end of the second year.

We have bred male MMTV-PyMT Spot14-/+ with female Spot14-/- mice to generate female MMTV-PyMT Spot14-/- experimental mice and have also bred MMTV-PyMT control mice. We have been and are currently monitoring tumorigenesis in all experimental and control mice. In all figures, we will refer to Spot14 as S14, as well as in the text beginning here. We have determined that the loss of S14 from PyMT tumors does not affect tumor latency (Figure 1A), but does affect other aspects of tumorigenesis.

Task 2 (Year 3 Months 1-6) We will monitor Control and Spot14-/- females for tumor formation and will harvest tissue when the tumor reaches 1 cm. Since the MMTV-PyMT model promotes tumor formation by 6 weeks, and most tumors reach 1 cm by 12 weeks, we expect to complete this aim by the middle of year 3.

We have monitored experimental and control mice for tumor formation and tumor growth rates. Approximately 1/3 of the MMTV-PyMT S14-/- females develop tumors with a fluid-filled cystic morphology. This was seen in the tumors themselves (Figure 1B) and also in the adjacent mammary glands (Figure 1C). The remaining MMTV-PyMT S14-/- mice developed tumors with reduced growth rates compared to MMTV-PyMT control mice (Figure 1D). The observations made in experimental mice support our hypothesis that Spot14 is required for tumor growth in MMTV-PyMT mice. We are finalizing studies in this model.

Task 3 (Year 2 Months 6-9) The preserved tumor samples collected from the recipient mice will be analyzed using qPCR, western blot, MRS, and immunohistochemistry. We anticipate completing this task by month 9 in the 3rd year.

We performed microarray analysis of solid tumors from MMTV-PyMT and MMTV-PyMT S14-/- mice. We found that 292 genes were significantly different between the two groups. GO Enrichment analysis of these genes revealed decreases in genes involved with cell cycle regulation and also with various metabolic pathways, including glycolysis/gluconeogenesis and the pentose phosphate pathway (Table 1). The differences in these genes have been validated by qPCR. This is consistent with the reduced growth rates of the tumors from PyMT/S14-/- mice.

Using GC-Mass Spectrometry, we evaluated the fatty acid profile of tumors from PyMT versus PyMT/S14-/- mice and from Neu and Neu/S14 mice. In the first group (PyMT versus PyMT/S14-/-), we found decreased levels of many fatty acids. The largest decrease in tumor fatty acids was seen in those with chain lengths $\leq$ 16 carbons, while more modest decreases were seen in fatty acids greater than 16 carbons. Conversely, compared to Neu tumors, those overexpressing S14 (Neu/S14) had higher levels of fatty acids, with the greatest enrichment seen in those with chain lengths $\leq$ 16 carbons (Figure 2A). Together, these data indicate that S14 regulates fatty acid synthesis in mammary tumors in vivo.

We are still in the process of analyzing the tumor tissue using western blot and immunohistochemistry analyses.

Milestone 1: Completion of Specific Aim 1 and preparation of manuscript for publication.

Although this was originally stated to be "milestone 1", because we have changed the SOW, we now expect this to be milestone 2, and to be completed by the end of the proposal funding period.

From SOW: Specific Aim 2: *Determine the effect of S14 overexpression on the onset, growth, metastasis and metabolism of mammary tumors arising in the MMTV-ErbB2 mice by generating MMTV-ErbB2, MMTV-Spot14 bitransgenic mice.*

We have completed this Specific Aim, and the results of these studies are described below, under each Task.

Task 1 (Year 1 Months 1-6): We will breed the MMTV-Spot14 and MMTV-c-ErbB2 mice to generate the single and bi-transgenic offspring that will be used in our studies. We anticipate completing this task by the end of the second quarter of the first year.

This Task has been completed. We have generated sufficient experimental and control mice to conduct powerful studies. The ErbB2 proto-oncogene is also called "Neu". Herein as we discuss results of the studies, we will refer to the control, MMTV-ErbB2 mice as "Neu" and the MMTV-ErbB2, MMTV-Spot14 bitransgenic mice as Neu/S14. The Neu/S14 mice are the experimental group. The remaining Tasks and Aim from the SOW are listed below. After that, please find the overall presentation of the results from the remainder of the analysis of Neu and Neu/S14 mice.

Task 2 (Year 1 Month 6-Year 2 Month 6): The MMTV-Spot14, MMTV-c-ErbB2, and MMTV-Spot14/MMTV-c-ErbB2 mice will be monitored for tumor onset and growth. The tumors will be evaluated as described in the project proposal and the animals will be sacrificed when the tumor reaches 0.5 cm in diameter. The tumor and other tissues will be harvested and preserved. We anticipate completing this aim in the second quarter of the second year.

Task 3 (Year 3 Months 1-6): The tumor and other tissue samples will be analyzed using qPCR, western blot, MRS, and immunohistochemistry. These analyses will also help us determine the effect of Spot14 on tumor cell metastasis, as lung tissues will be evaluated for the presence of mammary cancer cells. We anticipate completing this task by the second quarter of the third year.

From SOW: Specific Aim 3: *Determine the changes in the expression metabolic enzymes affected by gain or loss of Spot14 function in mammary tumors using microarray analysis.*

Task 1 (Year 1 Months 9-12): We will perform cDNA microarray analysis on tumor samples from the xenotransplantation of S14-/- and WT transformed mammary epithelial cells described in specific aim 1. We will also determine which pathways are affected by analyzing changes in the expression of specific genes associated with tumor cell metabolism. We anticipate completing this task by the last quarter of the first year.

Task 2 (Year 3 Months 1-12): We will perform cDNA microarray analysis on tumor samples from transgenic mice expressing ErbB2, Spot14, or both ErbB2 and Spot14 in the mammary epithelium. This will allow us to identify changes in the expression of specific metabolic pathways modulated by Spot14 using bioinformatics analysis. We anticipate completing this task and this aim by the last quarter of the third year.

Milestone 2: Completion of Specific Aims 2 and 3 and preparation of manuscripts for publication.

We have monitored tumorigenesis in Neu and Neu/S14 mice and found that Neu/S14 mice develop tumors significantly earlier than Neu control mice. These data are shown in Figure 3A. The log-rank p-value for this comparison is p=0.0034, with a hazard ratio of 0.2645 (Neu versus Neu/S14) and a 95% confidence interval (CI) of 0.1087 to 0.6435. We also quantified tumor multiplicity for Neu and Neu/S14 mice. This data is presented as number of tumors per mouse. Most mice had 1 or 2 tumors each, but some had more. We found no significant differences in tumor multiplicity between Neu and Neu/S14 mice (Figure 3B). These data suggest that Spot14 overexpression is sufficient to promote tumor formation in the presence of Neu oncogene activation, but does not act as an oncogene on its own, to increase tumor multiplicity. In other words, these data do not suggest that Spot14 can promote oncogenic transformation in the absence of Neu activation. These data support our hypothesis that Spot14 overexpression would stimulate tumor formation.

Tumor tissue was analyzed using a variety of methods. We began our studies by analyzing Neu signaling pathway activation in tumors from Neu and Neu/S14 mice. Western blot analysis of phosphorylated and total forms of Neu (ErbB2), ErbB3, Akt, and Erk showed us that kinase signaling was very heterogeneous within tumor groups, and was not different between tumor groups. Therefore, elevated Neu signaling in the established tumor could not explain the shortened tumor latency observed in Neu/S14 mice, compared to Neu controls (Figure 4). Using

immunohistochemistry (IHC), we found that Neu/S14 tumors had a significantly higher proliferative index, as measured by quantification of Ki67 staining, than Neu control tumors (Figure 5A and 5B). We also performed IHC analysis of cleaved Caspase-3 on tumors from both groups, which indicates apoptotic cells. We did not observe any differences between Neu and Neu/S14 tumors in cleaved Caspase-3 staining. In fact, many tumors did not have large apoptotic regions, and the images shown in Figure 4C represent apoptotic "hot spots" within the tumors.

Spot14 is known to play a role in regulating de novo fatty acid synthesis in the liver, adipose tissue, and the lactating mammary gland. Therefore, GC-Mass Spectrometry (GCMS) was used to evaluate tumor fatty acid content. We found a near universal increase in the content of many fatty acids, including non-esterified (NEFA, Free) fatty acids, and those contained in triglycerides, diacylglycerols, cholesterol esters, and phospholipid membranes (Total). These data are shown in Figure 6A and 6B. Table 2 lists each fatty acid chain length and saturation, with the measured amount in each group of tumors (average and SEM) and the p-value for each two-tailed, unpaired t-test. Those fatty acids that were significantly different between groups have a p-value less than 0.05 and are depicted in bold and italics. Notably, when we evaluated the differences in fatty acids by chain length groups, we found that S14 overexpression was associated with an increase in those with chain lengths less than or equal to 16 carbons (Figure 2B). This is consistent with observations made when S14 is lost from MMTV-PyMT mice. We used qPCR analysis to evaluate the expression of three de novo fatty acid enzymes in Neu and Neu/S14 tumors. ATP-Citrate Lyase (ACLY), Acetyl-CoA Carboxylase (ACC) and Fatty Acid Synthase (FASN) each play a critical role in providing substrates for the de novo synthesis of fatty acids. We did not find any differences in the expression of these genes between Neu and Neu/S14 tumors, suggesting that elevated enzyme levels do not explain the increase in tumor fatty acid content seen in Neu/S14 mice (Figure 6C, 6D, and 6E). Overall, these data suggest that Spot14 overexpression stimulates the synthesis of fatty acids in tumor cells, possibly by influencing the activity of the FASN enzyme, which is consistent with our hypothesis that Spot14 overexpression would promote de novo fatty acid synthesis in mammary tumors.

Lung tissue was collected from all tumor-bearing animals. To evaluate lung metastases, we cut 5 micrometer sections every 50 micrometers throughout the entire lung block for each animal. These sections were stained with H&E to visualize lung metastases. Dr. Paul Jedlicka, who is a board-certified pathologist in our department, assisted in analyzing the lung tissue for metastatic lesions. We quantified lung metastases by counting the number of mice with lung metastases out of the total number of mice examined (Figure 7A). Dr. Jedlicka also evaluated the primary tumors for signs of peri-tumoral lymphovascular invasion (LVI). Overall, we found that 1/15 Neu/S14 mice had metastasis of tumors to the lung, and we found that no tumors had signs of LVI. Conversely, 6/17 Neu mice had lung metastases. Evaluation of LVI revealed invading cancer cells in 3 Neu primary tumors. Of these 3 tumors, we already observed lung metastases in 2 of them, but 1 tumor had not yet metastasized to the lung at the time of sacrifice. If we

combine this data, we found that, overall, 7/18 Neu mice had invasive or metastatic tumors, while this was observed in only 1/15 Neu/S14 mice. The Fisher's Exact p-value for this difference is 0.046, meaning Neu/S14 tumors are significantly less likely to be invasive or metastatic than Neu tumors. These data are reported in Figure 7B, as an embedded table that depicts the differences in metastasis and invasion found between groups. Surprisingly, these data contradicted our hypothesis. We predicted that Spot14 overexpression would stimulate fatty acid synthesis in tumors, which would give them a *growth and metastatic* advantage. What we found, however, was that Spot14 overexpression did stimulate tumor growth, but the Neu/S14 tumors were not highly metastatic.

The reduction in metastatic and invasive ability of Neu/S14 tumors seemed to contradict the increase in tumor cell proliferation observed in this group. To identify novel differences in gene expression, we performed cDNA microarray analysis of Neu (N=13) and Neu/S14 (N=11) tumors. CEL files were produced from the Affymetrix Gene Atlas instrument. These CEL files were RMA normalized and Log2 transformed using Partek Genomic Suite software. Spreadsheets of combined data were then analyzed using Significance Analysis of Microarrays (SAM[1]) in Excel. Rather than using ANOVA, followed by a multiple-testing correction, SAM includes a false discovery rate (FDR) correction in the initial analysis, and reports data as fold change values (experimental versus control) and gives q-values. The SAM q-value is equivalent to a standard p-value. We created a list of genes that were different between Neu/S14 and Neu tumors at a q-value of 5% (0.05). At this significance level, we found that 456 genes were significantly elevated in Neu/S14 versus Neu tumors, while 26 were decreased. As an aside, I earned my PhD studying lactation in mouse models, so as I looked at the gene list, I noticed that many of the genes elevated in Neu/S14 tumors were also genes that I knew to be associated with the lactating mammary gland. To scientifically approach this, I took advantage of data from a separate ongoing study in our laboratory. For that separate study, we performed microarray analysis on enriched mammary epithelial cells from day 14 pregnant and day 4 lactating mice. I was able to cross-reference the genes elevated in lactation with the genes elevated in the Neu/S14 tumors, and found that 41% (188/456) of the genes increased in Neu/S14 tumors were also increased in the lactating mammary gland. The genes elevated at least 1.5 fold are listed in a table in Figure 8 (Figure 8A), which also includes one gene that was repressed in Neu/S14 versus Neu tumors. The genes listed in bold type are also elevated in lactation, and the genes with an asterisk (*) were validated using qPCR analysis. One of the most significantly elevated genes in Neu/S14 tumors was Elf5 (Figure 8B). Published studies have shown that Elf5 acts as a "master regulator" of mammary alveolar cell expansion during pregnancy and lactation[2]. In fact, mice lacking Elf5 in the mammary gland fail to lactate. Elf5 is thought to act downstream of prolactin signaling during lactation to participate in the induction of differentiation-associated genes during lactation. Additionally, in human breast cancer cells, Elf5 is necessary to mediate the proliferative effects of progesterone[3]. Together, these studies suggest that Elf5 can promote both proliferation and differentiation of the mammary gland. These data were quite surprising, but also explained the decreased metastatic ability of Neu/S14 tumor cells, compared to Neu

controls. Lactation represents a time when the mammary epithelium goes through the process of terminal differentiation. Among other significantly elevated genes was the epithelial marker, keratin 18 (Figure 7C). Finally, Elf5 was shown to promote the expansion of a population of alveolar epithelial cells that lacks the cell surface marker, CD61 or B3-integrin (Itgb3). We found that Itgb3 expression in Neu/S14 tumors trends towards being significantly lower than Neu tumors (Figure 8D). Serum prolactin levels have been measured in MMTV-S14 and wild type mice, and have shown that S14 overexpression does not influence the levels of circulating prolactin. Therefore, the effects of S14 on tumor differentiation are likely independent of elevated circulating prolactin (Figure 8E). Differentiated cancer cells are thought to be less metastatic than undifferentiated cells.

One study has been published that demonstrated an increased risk for breast cancer patient death associated with S14 protein levels[4]. Specifically, these investigators found that S14 protein was directly correlated to increasing tumor grade, and that lymph-node positive breast cancers expressing high S14 were more likely to kill the patient than those with low S14. There are several points to make regarding this study. First, the association of S14 with tumor grade and with patient outcome suggests that S14 is not an independent predictor of death from breast cancer. High tumor grade is known to correlate with reduced survival of breast cancer patients. Second, the investigators analyzed lymph-node positive tumors, which means, to some extent, these cancers were already invasive. Third, the antibody used for IHC analysis of S14 in this study was made by the investigators, and they have not made the antibody available for other investigators to use, so unfortunately, we cannot repeat their study in an independent breast cancer sample set. To determine if S14 gene expression was correlated with patient outcomes for breast cancer, I analyzed publicly available microarray data as an alternative to performing IHC analysis on breast tumor samples. I obtained gene expression datasets of primary human breast tumors, either from Oncomine[5] or the NCBI Gene Expression Omnibus (GEO). Each of these datasets had expression information for S14 (called THRSP on the array platforms), and also had some sort of patient outcome data. This included either disease-free survival, where disease is defined as cancer recurrence or cancer metastasis, or disease-specific survival, which is defined as death from breast cancer, and will be referred to as “overall survival”. The details of these datasets are presented in Table 5. Initially, I found the median expression level for S14 in each dataset, and all patients with above median expression were called “high S14” expressing, while those below the median were called “low S14” expressing. This allowed me to convert S14 expression to a categorical variable, which meant the datasets could be combined. When I analyzed the outcomes of these patients using a Kaplan-Meier approach, with a Log-rank (Mantle-Cox) test, I found that there were no differences in patient outcome correlating with S14 expression (data not shown). To more precisely separate the cases into high and low S14 expressing groups, I sorted the datasets individually by S14 expression, and took the upper and lower 25% of the samples to use for analysis, giving me the more extreme high and low expressing tumors. Performing the statistical analysis on these groups, representing “high S14” and “low S14”, respectively, showed me that patients with high S14 expressing tumors were

significantly less likely to experience disease recurrence or metastasis, and were also significantly less likely to die from disease, compared to patients with low S14 expressing tumors (Figure 9). These data are exciting, and support the observations seen in our mouse model. Together, these data suggest that S14 overexpression is associated with a particular tumor cell type that is well differentiated and is not likely to metastasize. The question remained, then, does S14 promote differentiation, or does is simply permit the expansion of a differentiated cell type within the pre-tumorigenic mammary gland?

To address this question, I used mouse mammary epithelial cells, which were engineered to express S14 under the control of a doxycycline-inducible (dox) promoter. Dox addition results in a dose-dependent increase in S14 expression levels in this model (Figure 10A). S14 was induced for 24 hours, and then cells were monitored for proliferation for an additional 4 days. These cells were compared to cells that were not treated with dox. We found that S14 overexpression in cultured mammary epithelial cells significantly increased proliferation over the course of the 4 day assay, compared to cells without S14 induction (Figure 10B). RNA was harvested from cells with and without S14 expression and qPCR analysis was performed on several differentiation-associated genes that were identified using microarray analysis of mouse tumors (see Table 4). Analysis of Elf5, Keratin 18, and Butyrophilin 1a1 (Btn1a1) showed that S14 induction did not increase the expression of these genes, known to be associated with mammary epithelial cell differentiation (Figure 11). We also assayed the levels of Cck, Csn1s2a, and Lao1, all of which were increased in Neu/S14 compared to Neu tumors, but these genes were not expressed in CIT3 cells, and showed no increase with S14 induction (data not shown). Together, these data suggest that S14 promotes the proliferation of cells in which it is expressed, but does not promote differentiation of mammary epithelial cells, per se.

Based on these studies, we predicted that S14 overexpression would promote proliferation in the mammary gland of MMTV-Neu mice before tumors form. To determine if this was the case, we performed whole-mount analysis on mammary glands from age-matched (10 months) and diestrus-staged Neu and Neu/S14 females. The mammary gland is known to experience cycles of proliferation in response to the elevated progesterone levels that occur during the estrus cycle (www.mammary.nih.gov). Thus, when analyzing mammary glands from nulliparous, cycling female mice, it is important to monitor the estrus cycle and harvest tissue from females that are in the same stage of their cycles. We found that Neu/S14 mammary glands displayed hallmarks of hyperplastic alveolar nodules (HAN), which were not seen in Neu control glands (Figure 10C). These data suggest that S14 overexpression in MMTV-Neu mice is sufficient to stimulate proliferation of the mammary epithelium.

From these results we have created a model that predicts a role for S14 in breast cancer (Figure 12). In the normal mammary gland, S14 expression increases beginning in late pregnancy and is dramatically elevated in the epithelial compartment during lactation. At this time, the mammary gland is being instructed by prolactin signaling. We predict that S14-mediated fatty acid synthesis supports milk production during lactation, as that is what prolactin is guiding the

mammary epithelium to do. Based on the normal mammary gland, we predict that high S14 expression is associated with a well-differentiated epithelial cell type. When S14 is highly expressed in the absence of prolactin, and in the presence of oncogenic signaling, for example, we hypothesize that it stimulates fatty acid synthesis, through a similar mechanism that is seen during lactation, but these cells are not being guided to make milk. Instead, we hypothesize that these fatty acids are signaling to the cell to proliferate, which shortens the process of tumor formation in the presence of the Neu oncogene. This results in the more rapid formation of a tumor, but because a very particular type of cell is proliferating, these tumors are not metastatic. Instead they are well differentiated, and express many genes found in the lactating mammary gland. The results of this study are being prepared for publication, and we anticipate submitting a manuscript to Cancer Research by the end of October.

The remainder of this grant period will be focused on the studies in MMTV-PyMT mice with and without S14 expression. Initial studies performed in these mice suggest that S14 expression is required for tumor cell proliferation, which is consistent with results obtained in Neu/S14 bitransgenic mice.

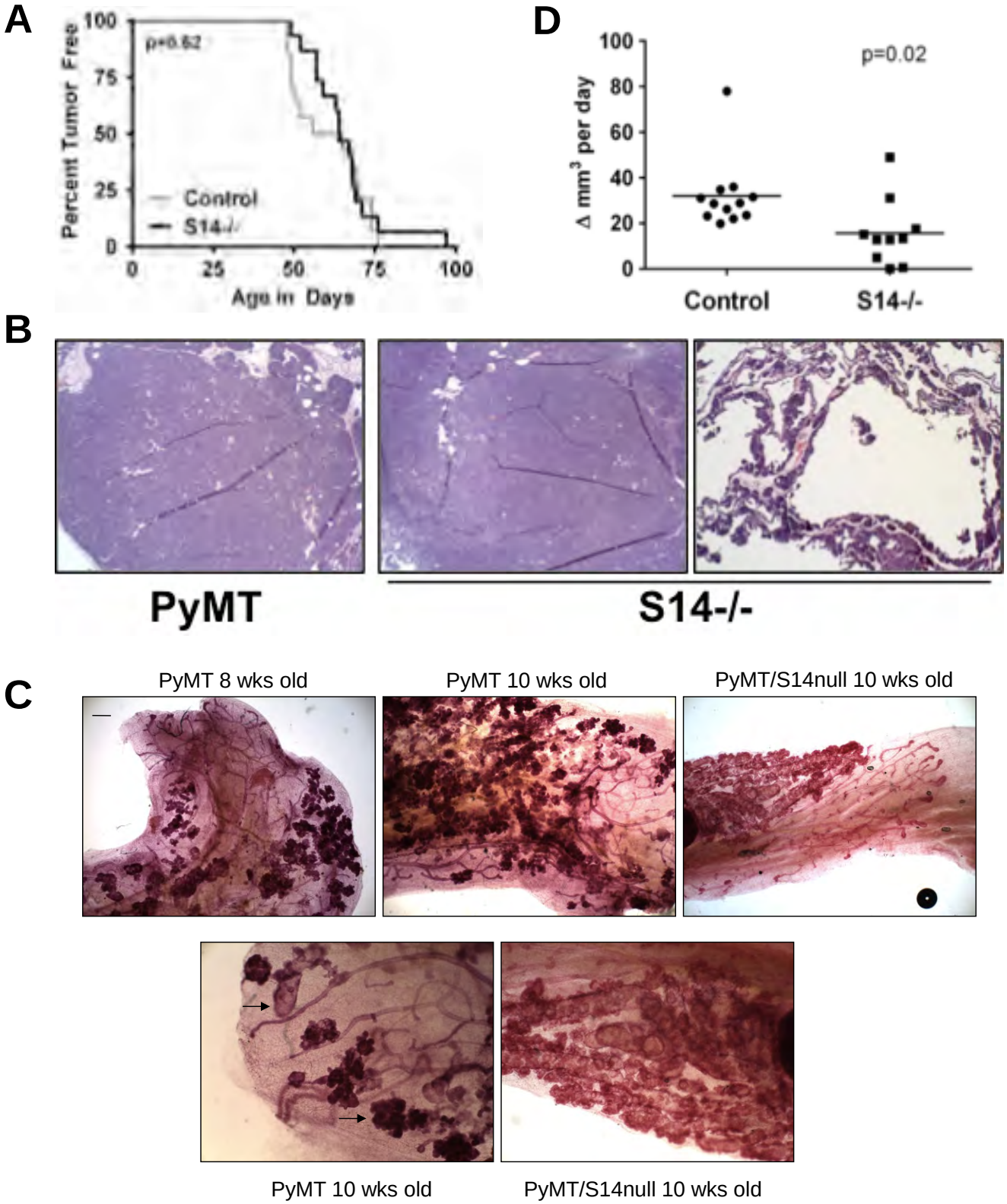


Figure 1. A. Kaplan Meier Survival Curve. PyMT (Control, N=14) and PyMT S14-/- (S14-/-, N=15) mice. HR 0.8165, 95% CI 0.39-1.7. p=0.62 (Log-rank). B. H&E stained images of tumors from PyMT and PyMT S14-/- mice. C. Whole mount analysis of mammary glands from MMTV-PyMT and MMTV-PyMT S14-/- mice. Scale bar=500 micrometers

KEGG Pathway Enrichment	Enrichment Score	Enrichment p-value
GLYCOLYSIS_GLUCONEOGENESIS	6.372	0.002
PENTOSE_PHOSPHATE_PATHWAY	5.022	0.007
CELL_CYCLE	4.546	0.011
FRUCTOSE_AND_MANNOSE_METABOLISM	4.135	0.016
CELL_ADHESION_MOLECULES_CAMS	3.522	0.030
REGULATION_OF_ACTIN_CYTOSKELETON	3.076	0.046
GALACTOSE_METABOLISM	2.783	0.062
P53_SIGNALING_PATHWAY	2.493	0.083
RENAL_CELL_CARCINOMA	2.317	0.099

Table 1. GO Enrichment analysis of tumors from PyMT and PyMT S14-/- mice. The pathways are presented as those enriched in PyMT S14-/- versus PyMT tumors.

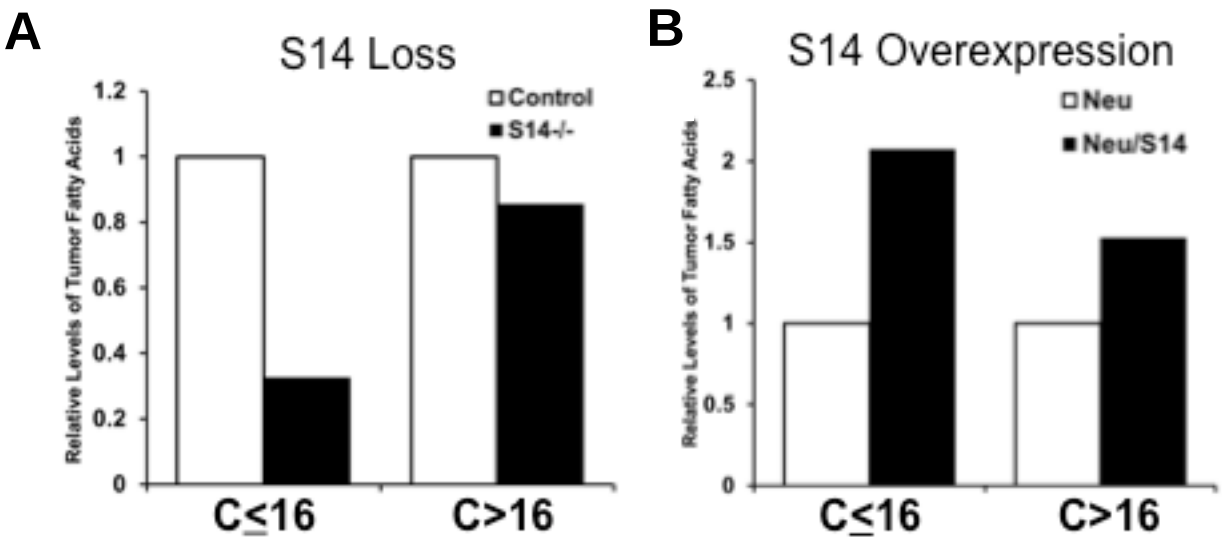


Figure 2. A. Relative levels of fatty acis by chain length in PyMT (Control) and PyMT S14-/- mice. **B.** Relative levels of fatty acids by chain length in Neu versus Neu/S14 tumors.

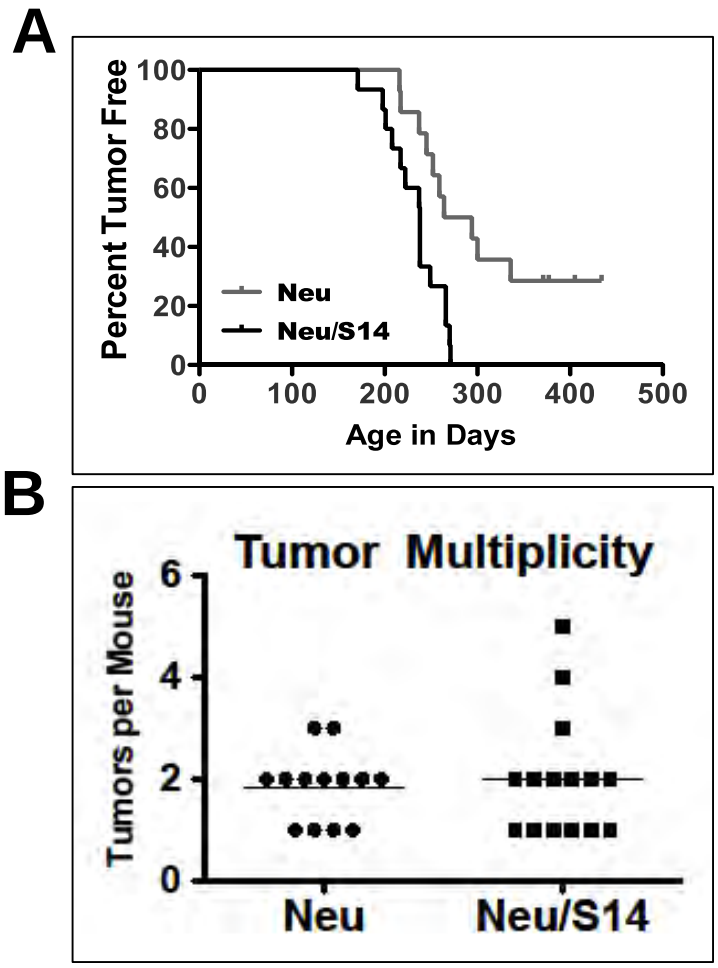


Figure 3. Analysis of tumorigenesis in Neu and Neu/S14 mice. A) Kaplan-Meier survival curves of tumor latency in Neu and Neu/S14 mice. Log-rank p-value = 0.0034, showing S14 expression is associated with a significantly shorter tumor latency. B) Tumor multiplicity in Neu and Neu/S14 mice, expressed as number of tumors per mouse.

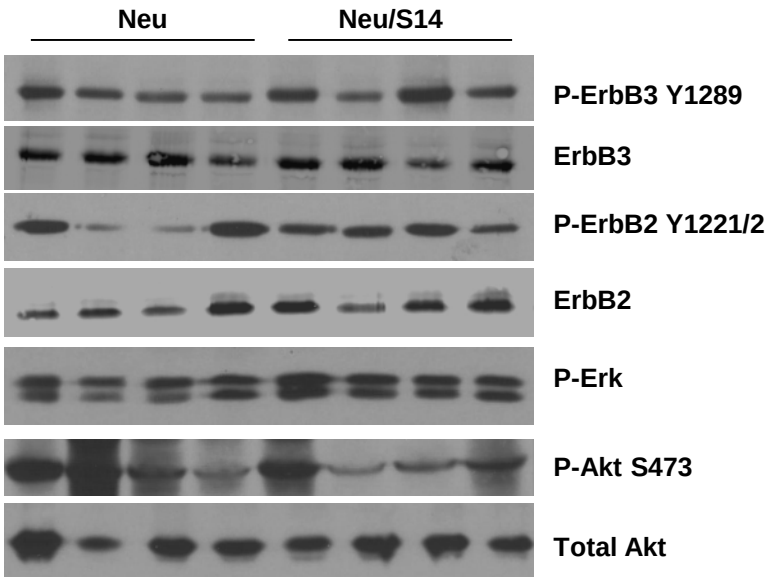


Figure 4. Western Blot analysis of tumors from Neu and Neu/S14 mice. Differences were not detected in signaling pathway activation between Neu and Neu/S14 tumors. Signaling molecule activation was highly variable within tumor groups.

Fatty Acid Chain Length and Saturation	Total Fatty Acids (ng per mg tissue)		Ratio Neu/S14 to Neu	p-value	Non-esterified Fatty Acids (ng per mg tissue)		Ratio Neu/S14 to Neu	p-value
	Neu	Neu/S14			Neu	Neu/S14		
10:0	0.209	0.688	3.3	0.104	0.021	0.034	1.6	0.142
12:0	0.165	0.235	1.4	0.213	0.025	0.042	1.7	0.117
14:0	0.635	1.153	1.8	***0.027***	0.145	0.238	1.6	***0.004***
14:1	0.029	0.098	3.4	***0.013***	0.013	0.023	1.8	***0.002***
16:0	4.248	8.308	2.0	***0.012***	0.862	1.706	2.0	***0.003***
16:1	0.479	1.257	2.4	***0.017***	0.077	0.211	2.7	***0.003***
18:0	4.026	5.240	1.3	0.099	0.814	1.260	1.5	***0.019***
18:1	4.182	7.349	1.8	***0.028***	0.721	1.619	2.2	***0.005***
18:2	2.713	5.725	2.1	***0.025***	0.421	1.056	2.5	***0.007***
18:3	0.019	0.016	0.8	0.218	*nd*	*nd*	-	-
20:4	4.686	3.576	0.8	0.230	0.307	0.400	1.3	0.069

Table 2. GCMS analysis of fatty acids in tumors from Neu and Neu/S14 mice. The fatty acid chain lengths and saturations, the average content of fatty acids in Neu (N=7) and Neu/S14 (N=7) tumors, and the ratio of each fatty acid average in Neu/S14 versus Neu tumors are listed. Two-tailed t-tests were used to determine if differences between groups were statistically significant. P-values in bold and italics are significant ($p<0.05$).

Metabolite	Neu		Neu/S14		pvalue
	Average	SEM	Average	SEM	
Lactose	**1.31**	**0.23**	**1.99**	**0.23**	***0.05***
GPC	2.65	0.26	2.43	0.37	*0.35*
tCholine	0.82	0.19	0.50	0.06	*0.10*
GSH	0.34	0.21	0.10	0.01	*0.18*
DMA	0.25	0.06	0.17	0.02	*0.15*
Glutamate	2.77	0.86	3.79	0.61	*0.21*
Acetate	0.26	0.07	0.28	0.05	*0.42*
OH-Butyrate	**3.36**	**0.42**	**1.58**	**0.68**	***0.05***
Acetyl-CoA	0.28	0.12	0.24	0.06	*0.42*
Val, Leu. Ile	3.03	0.65	4.20	0.10	*0.08*

Table 3. NMR analysis of aqueous metabolites in tumors from Neu and Neu/S14 mice. Perchloric acid was used to extract aqueous metabolites from frozen tumor tissue. The amounts of each metabolite are per mg of tumor tissue. The only significant differences between groups were observed in lactose and OH-butyrate. Lactose is a milk disaccharide, which is produced by differentiated alveolar epithelial cells. Student's unpaired two-tailed t-tests were used to compare metabolite levels between groups.

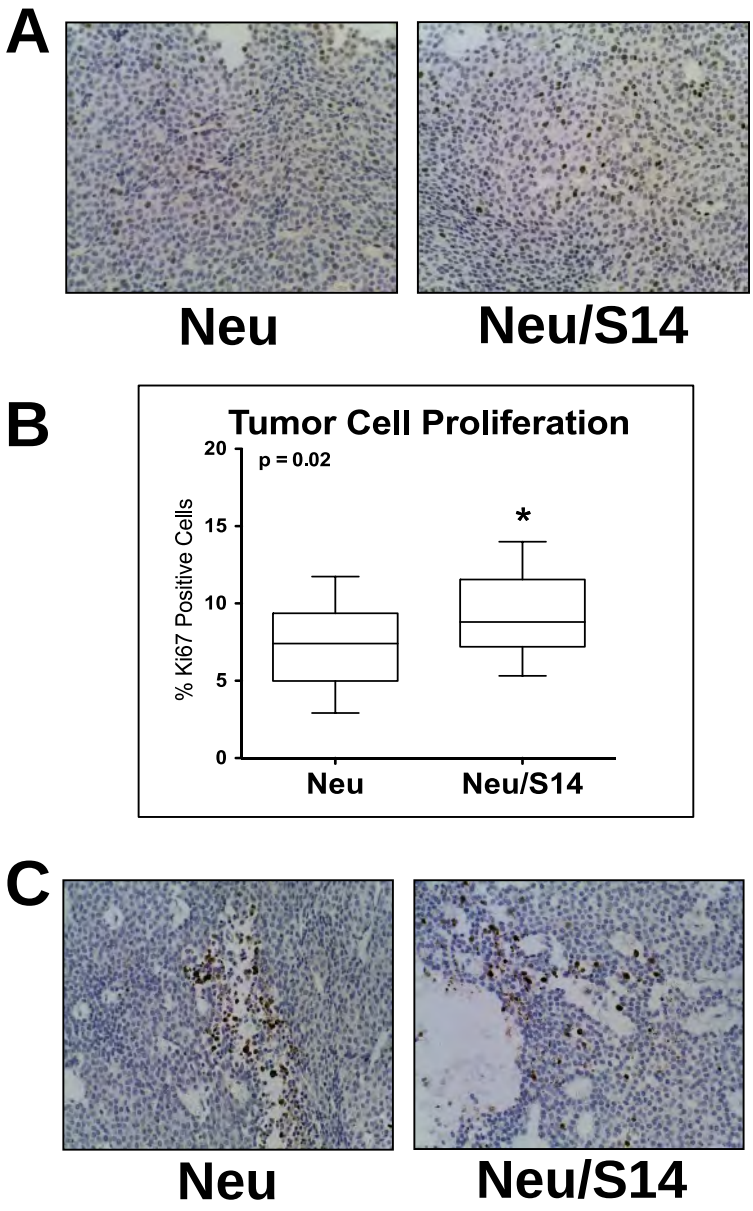


Figure 5. Analysis of proliferation and apoptosis in Neu and Neu/S14 tumors. IHC was used to analyze Ki67 (A) in tumors from both groups. The number of Ki67 positive nuclei out of total nuclei per 20X field from 5 images per tumor of 5 tumors per group was counted and is reported in (B). A student's unpaired, two-tailed t-test was used, and showed a pvalue of 0.02 for the difference in proliferation between groups. IHC analysis was also used to evaluate cleaved Caspase-3 (C) as a marker of apoptosis. Overall, apoptosis was low in tumors from both groups and did not appear to be different.

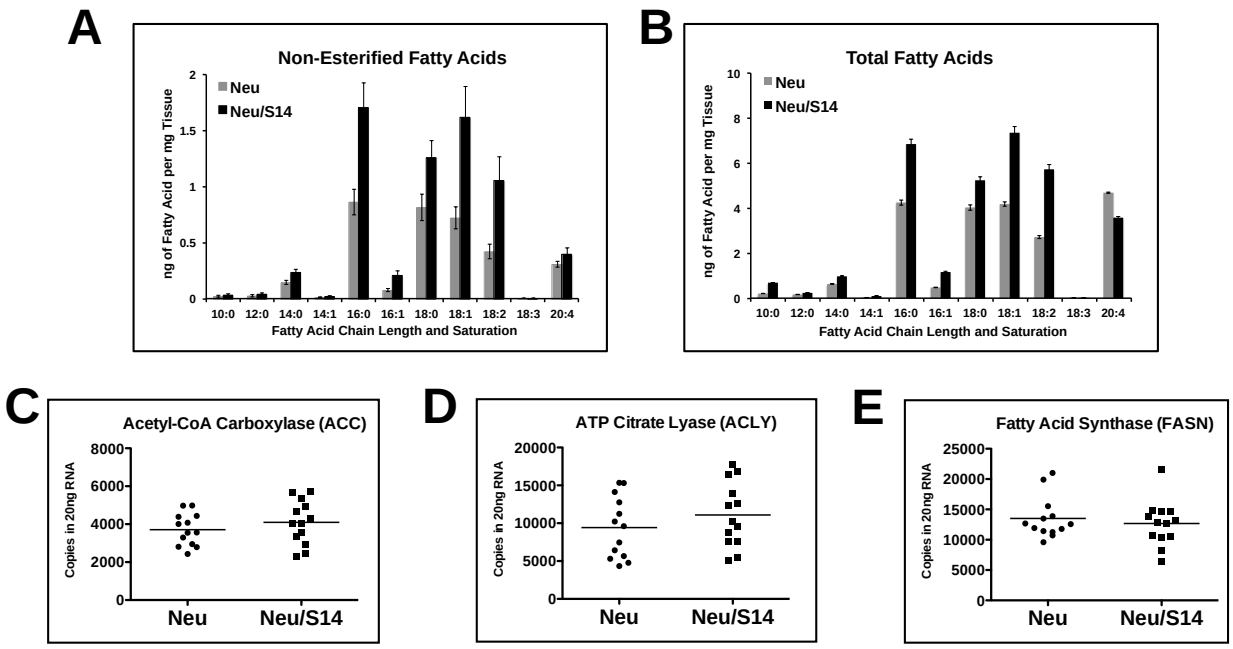
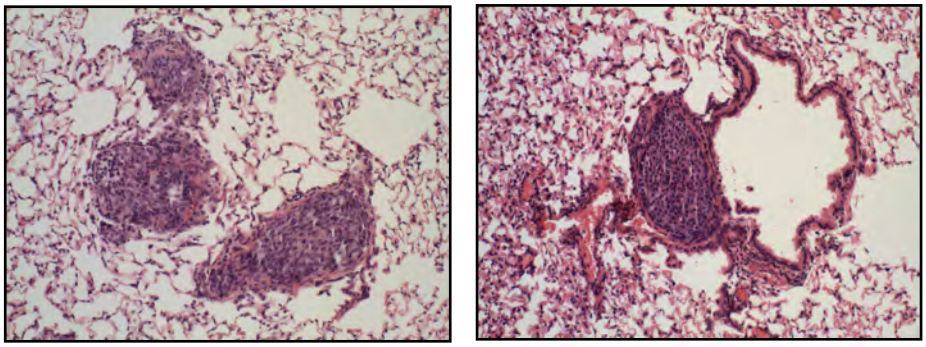


Figure 6. Analysis of fatty acids and de novo fatty acid synthesis enzymes in Neu and Neu/S14 tumors. GCMS was used to analyze non-esterified or free fatty acids (A) and total fatty acids (B) in mouse tumors. Nearly all fatty acids analyzed were elevated in Neu/S14 versus Neu tumors. QPCR analysis of de novo fatty acid synthesis pathway enzymes Acetyl-CoA Carboxylase (ACC, C), ATP-Citrate Lyase (ACLY, D), and Fatty Acid Synthase (FASN, E), showing no differences in expression between groups.

A



Neu Neu/S14

B

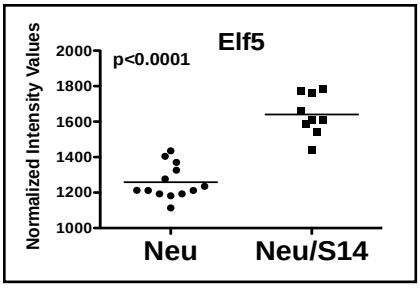
Group	Invasive or Metastatic/total
Neu	7/18 (38.9%)
Neu/S14	1/15 (6.7%)

Figure 7. Analysis of invasion and metastasis in Neu and Neu/S14 mice. A) Representative metastatic lesions in the lungs of Neu (left) and Neu/S14 (right) mice. B) Quantification of mice with invasive or metastatic tumors out of total mice examined. Fisher's Exact test was used to determine that S14 overexpression is associated with a significantly reduced risk of invasion and metastasis (p=0.046).

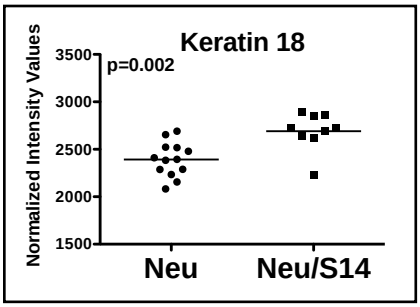
A

Neu/S14 > Neu	
Gene	**Fold**
*** Lao1**	**3.86**
*** Csn1s2a**	**3.52**
*** Cck**	**3.28**
*** Btn1a1**	**2.93**
Prol1	**2.68**
C87977	**2.60**
*** Arg2**	**2.43**
Cd36	**2.24**
Cytip	**2.16**
Tomm5	2.03
Oog1	**1.98**
Spink5	1.97
LOC100503984	1.96
Olah	**1.90**
Snord61	1.89
Pcdhb17	**1.87**
Rny1	1.82
Gm13023	**1.81**
Ust	**1.75**
* Lef1	1.75
Sdr16c5	**1.74**
Mfsd2a	**1.73**
Dpp4	**1.72**
Entpd1	1.65
Slc2a9	1.63
Rgs1	1.62
Sectm1a	**1.61**
*** Olfr234**	**1.61**
Ada	1.60
*** Thrsp**	**1.57**
Smr2	**1.55**
Gulp1	**1.54**
Chn2	1.53
Rnasel	1.53
Tcfcp2l1	**1.50**
* Cpeb2	0.61

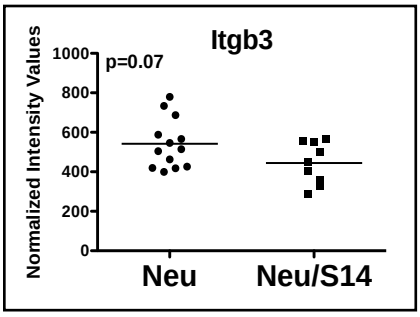
B



C



D



E

Genotype	Prl (ng/mL)
Wild Type	23.6 ± 3.3
MMTV-S14	18.4 ± 7.6

Figure 8. Microarray analysis of Neu and Neu/S14 tumors reveals differences in cellular differentiation. A) Table of genes elevated in Neu/S14 versus Neu tumors greater than 1.5 fold, and including one gene decreased in Neu/S14 versus Neu tumors. Genes in bold are also elevated during lactation, and the asterisk (*) denotes genes whose expression changes were validated using qPCR. Increases in differentiation-associated genes, Elf5 (B) and Keratin 18 (C) were found in Neu/S14 tumors. The cell surface marker, CD61 or B3-integrin (Itgb3) represents an alveolar precursor, and when expression is lost, cells are thought to differentiate (D). E) Serum prolactin levels are not different between MMTV-S14 and wild type mice.

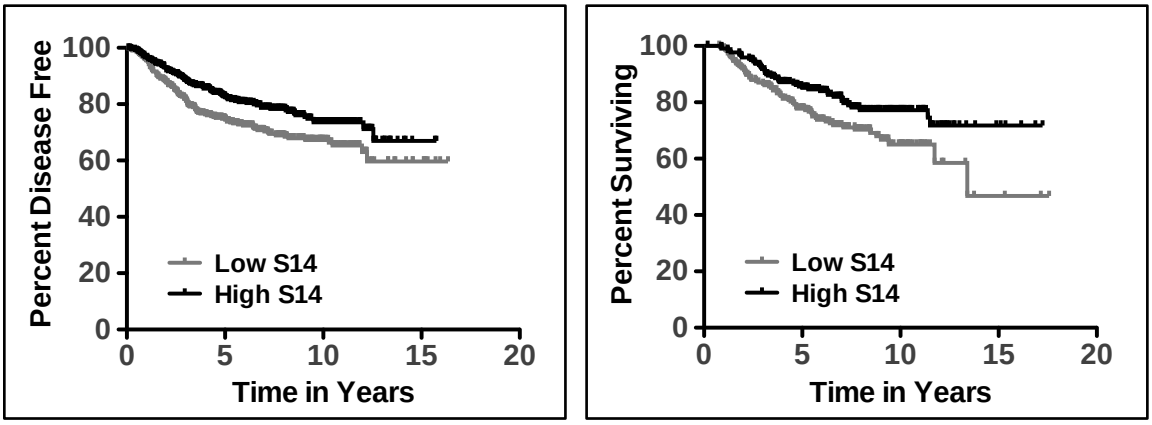


Figure 9. Kaplan-Meier survival curves from primary human breast cancer data. Details of analysis are described in the text above. Patients with high S14 expressing tumors are less likely to experience disease recurrence or metastasis (left; Log Rank pvalue 0.0073) or to die from disease (right; Log Rank pvalue 0.013) compared to patients with low S14 expressing tumors.

GEO ID	Total Tumors	Low S14	High S14	Platform	PMID	Analysis Performed
GSE19615	115	29	29	HG-U133P2	20098429	Disease-Free Survival
GSE6532	414	102	91	HG-U133A, HG-U133B, HG-U133P2	20479250, 18498629, 17401012	Disease-Free Survival
GSE20685	327	82	82	HG-U133P2	21501481	Disease-Free Survival, Overall Survival
GSE1456	159	40	40	HG-U133A, HG-U133B	16280042	Disease-Free Survival, Overall Survival
N/A (Available from Oncomine)	295	73	73	Printed Microarray	12490681, 11283592	Overall Survival
GSE4922	249	63	63	HG-U133A, HG-U133B	17079448	Disease-Free Survival
GSE21653	266	60	59	HG-U133P2	20490655, 22110708	Disease-Free Survival
GSE22226	130	33	33	Agilent Human Genome 44K	22198468	Disease-Free Survival, Overall Survival
Total Tumors	1955	482	470			
Disease-Free	1660	409	397			
Overall	911	228	228			

Table 4. Details of datasets used for survival analysis. Data were obtained from Oncomine or from the NCBI Gene Expression Omnibus (GEO). All data were normalized within datasets. The details of the analysis are described in the above text.

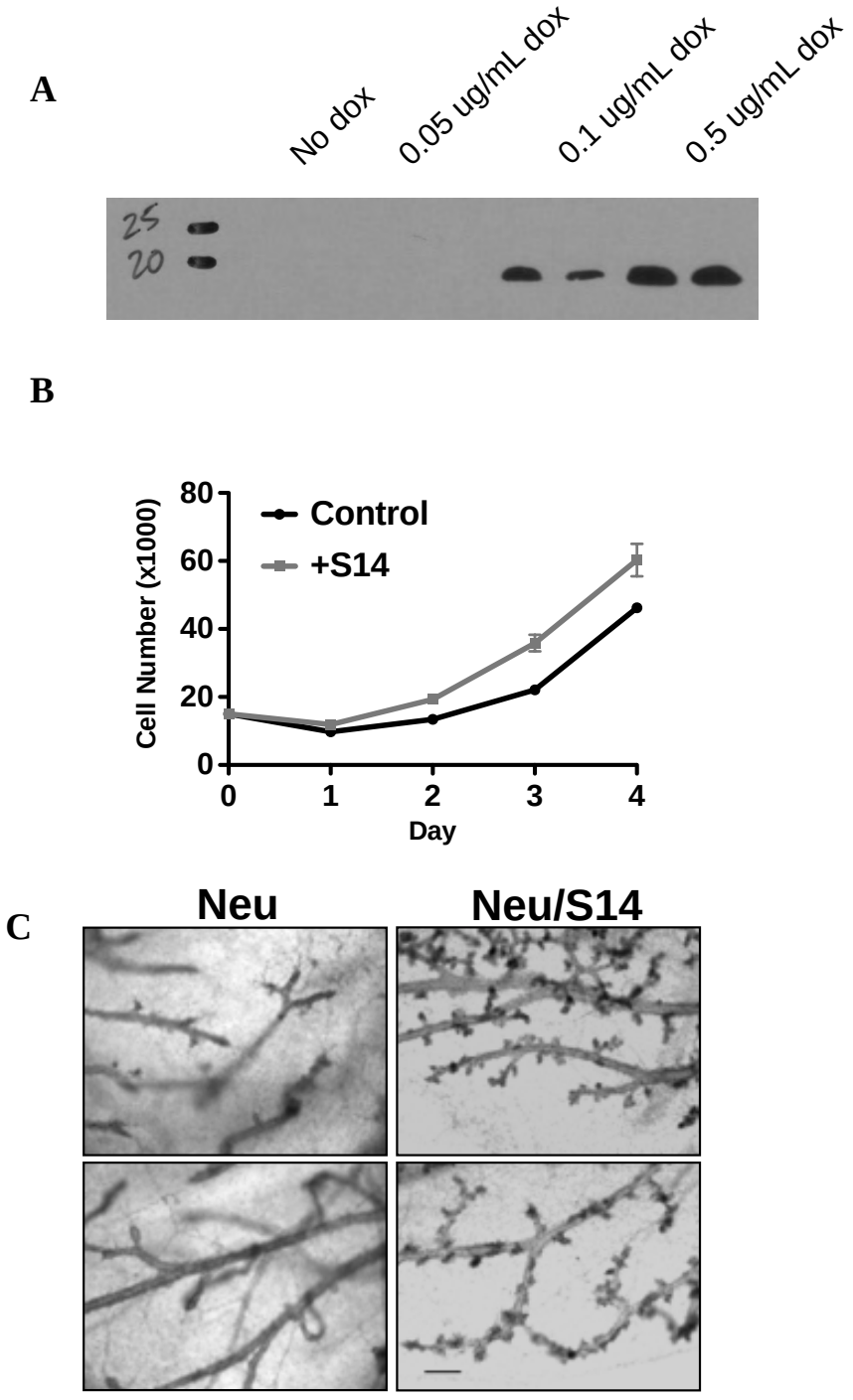


Figure 10. Analysis of proliferation in normal mammary epithelial cells and non-tumor bearing mammary glands. A) Western blot analysis of HA protein levels following doxycycline treatment of CIT3 cells. B) Proliferation assay of CIT3 mammary epithelial cells with and without S14 expression. Beginning at day 2, the S14 expressing cells are significantly higher than controls cells. Student's two-tailed t-test was used to compare control versus S14 cells at each day. Days 2-4 pvalue <0.001. C) Whole mount images of mammary glands from Neu and Neu/S14 mice. Note the alveolar nodules present in Neu/S14 glands.

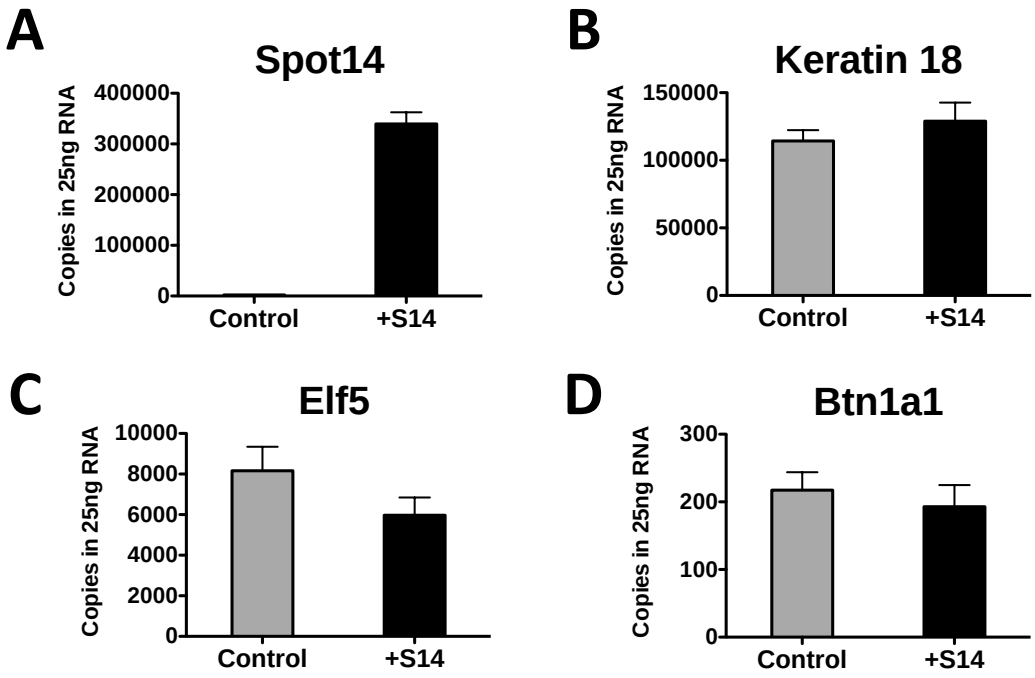


Figure 11. Q-PCR analysis of differentiation-associated genes in CIT3 cells with and without S14 expression. CIT3 cells were treated with vehicle or doxycycline, to induce S14, for 48 hours. RNA was isolated and qPCR analysis was performed for Spot14 (A), Keratin 18 (B), Elf5 (C), and Btn1a1 (D). S14 did not stimulate the elevation of differentiation genes in CIT3 cells. Cck, Lao1, and Csn1s2a, three genes that were elevated in Neu/S14 versus Neu tumors, were also analyzed but were not detectable in CIT3 cells (data not shown).

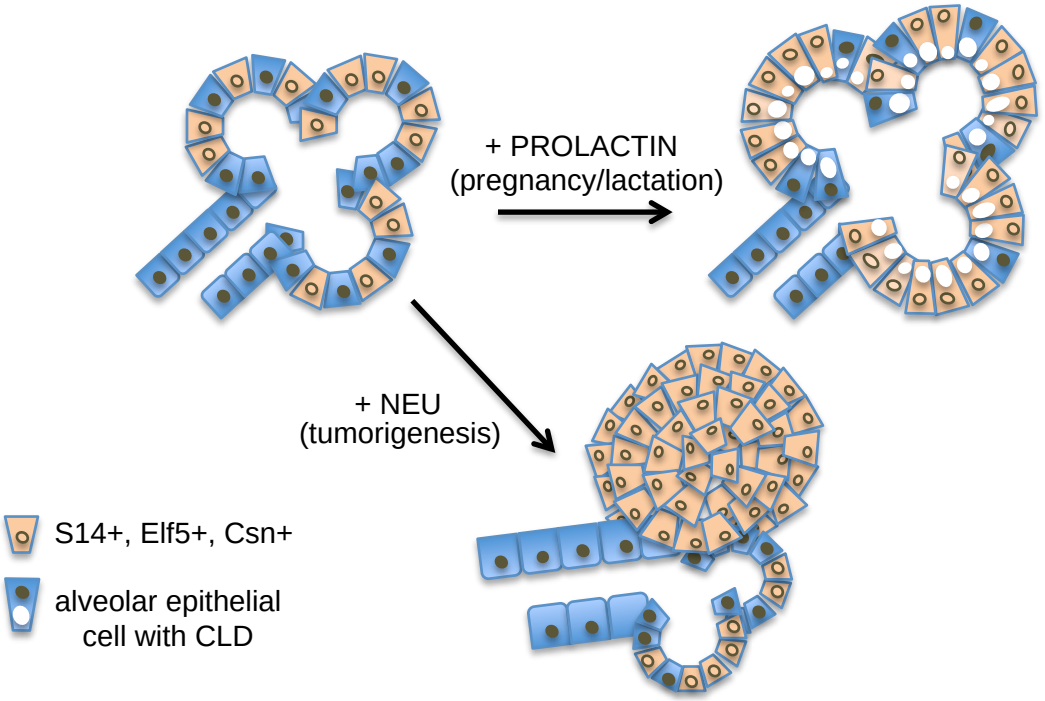


Figure 12. Proposed model of Spot14 action in the mammary gland and breast cancer. Based on our studies, we predict that Spot14 is expressed in a differentiated cell type within the mammary gland; a cell that also expresses Elf5 and Csn genes. In these cells, Spot14 elevates fatty acid synthesis. Under normal developmental circumstances, particularly during pregnancy and lactation, the mammary tissue is exposed to prolactin, which instructs the cells to produce milk from available fatty acids. In the context of oncogenic signaling, however, we expect that the Spot14-mediated increase in fatty acid synthesis gives these differentiated cells a proliferative advantage, which causes the early emergence of tumors. Because of their gene expression profiles, we predict that these tumors are not highly metastatic.

Key Research Accomplishments

- We have generated MMTV-Neu/MMTV-Spot14 (Neu/S14) mice and have completed the study on mammary tumorigenesis.
- From the above model, we have demonstrated a role for Spot14 in stimulating the formation of Neu-induced mammary tumors and enhancing cell proliferation within those tumors.
- We have performed NMR, GC-mass spec, and microarray analyses on tumors from experimental and control groups and have shown that Neu/S14 tumors are well-differentiated, as they display hallmarks of the lactating mammary gland.
- We have shown that Neu/S14 tumors have elevated fatty acid contents, but that this is not sufficient to increas metastasis as we had previously suspected.
- I have learned to perform bioinformatics analysis of public human tumor datasets and metadata and have used these tools to support the studies performed in our mouse model.
- We have generated MMTV-PyMT/Spot14-null mice to satisfy the remaining part of this proposal and have performed analyses on these mice. These studies have been combined for publication and will be submitted to Oncogene.

Reportable Outcomes

A manuscript is in preparation describing these studies. We anticipate submitting this work to Oncogene this month.

The results of these studies were presented at the Mammary Gland Biology Gordon Research Conference in June 2013.

We have generated MMTV-Neu/MMTV-Spot14 bitransgenic mice that can be made available to other researchers.

We have generated MMTV-PyMT S14-/- mice that can be made available to other researchers.

From this tumor study, we have performed microarray analysis of tissues from Neu and Neu/S14 mice. These microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database for access by the community. We have also performed microarrays analysis of tissues from PyMT and PyMT S14-/- mice and will deposit these data into GEO.

Based on these studies, our laboratory has acquired transgenic mice in which expression of Fatty Acid Synthase (FASN) can be eliminated in developing tumors. The studies described here were used as background in an application for the DoD BCRP Idea Award mechanism this year, in which we proposed to perform tumor studies in mice with and without the ability to synthesize fatty acids de novo in cancer cells.

Conclusions

Based on what we knew about the role of S14 in regulating de novo fatty acid synthesis and on its published role in breast cancer outcome, we hypothesized that S14 overexpression would elevate fatty acid synthesis in cancer cells, which would stimulate tumor formation, tumor growth, and tumor metastasis. To test this hypothesis, we generated a transgenic mouse model that expresses S14 in the mammary epithelium and crossed that model with an existing model of mammary tumorigenesis, the MMTV-Neu mouse. We have shown that S14 overexpression shortens tumor latency, stimulates proliferation of tumor cells and non-tumor epithelial cells, yet does not promote tumor metastasis. Using gene expression profiling, we identified that Neu/S14 tumors were more differentiated than Neu tumors, which explains their decreased metastatic activity. We have also generated a mouse model that lacks S14 expression in mammary tumors. Using this model, we have shown a requirement of S14 for rapid tumor growth and fatty acid synthesis. We analyzed human breast tumors for S14 expression and associated high levels of S14 with a favorable outcome. This is a clear discrepancy with what was published for S14, however our analysis included several hundred primary breast tumors from multiple datasets.

Why does this matter?

The conclusions of our studies are that elevated de novo fatty acid synthesis, per se, may be sufficient to stimulate cell proliferation and tumor growth, but does not appear to be sufficient for tumor metastasis. Rather, it appears that the metastatic ability of a tumor is hard-wired into its gene expression profile, and may not be so easily influenced by changes in tumor metabolism. This conclusion is supported by public human breast tumor microarray data, which shows that high S14 expression is protective from disease metastasis and from death due to disease. These studies suggest that S14 may be an important marker of differentiation status of human breast tumors that could give insight into the type of outcome a patient might experience.

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